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ENZYMATIC HYDROLYSIS OF CELLULOSE TO GLUCOSE

A REPORT ON THE NATICK PROGRAM

By

Enzyme and Biochemical Engineering Group
Environmental Sciences and Engineering Division
Science and Advanced Technology Laboratory

September 1981

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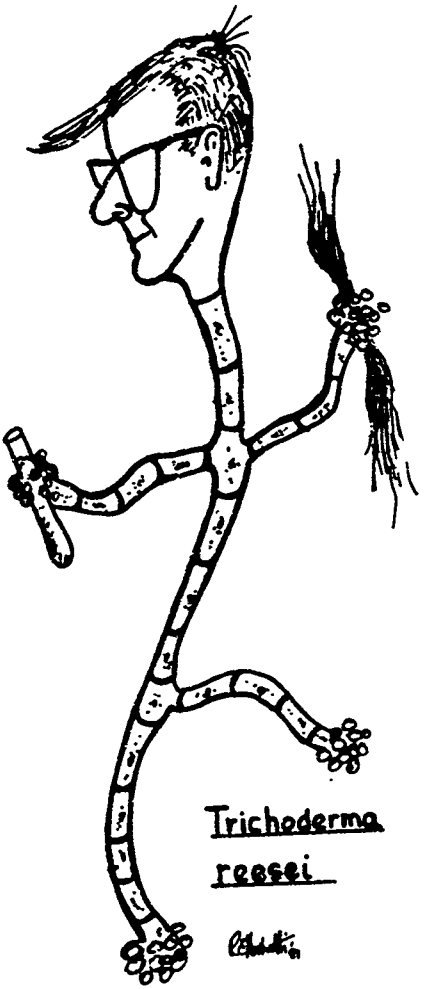
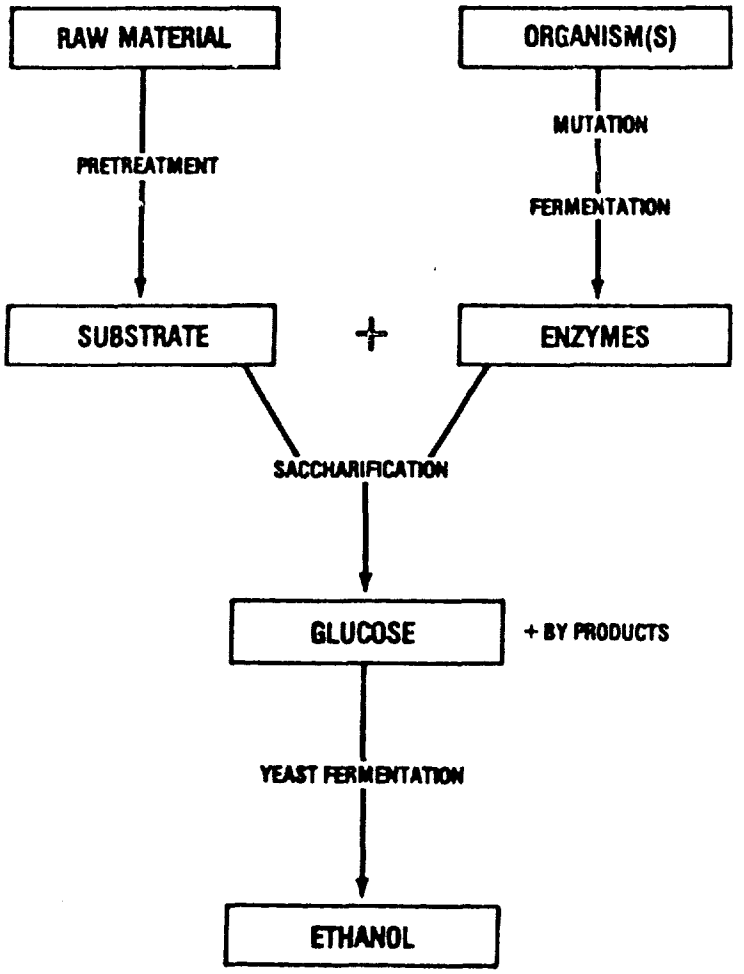

Trichoderma
reesei

ENZYMATIC SACCHARIFICATION OF WASTE CELLULOSE



Introduction

The roots of the Natick work on Enzymatic Hydrolysis of Cellulose to Glucose go back to the classic paper of Reese, Siu and Levinson in 1950 (1) which reported that many fungi grow well on cellulose and degrade it but the culture filtrates usually do not hydrolyze insoluble cellulose (although they frequently hydrolyze soluble cellulose derivatives such as carboxymethyl cellulose). To explain this the authors proposed that several enzymes are required to degrade crystalline cellulose and filtrates of cellulose cultures may not contain all of these. This is the famous C_1 C_x hypothesis.

$$\text{Crystalline Cellulose} \xrightarrow{C_1} \text{Reactive Cellulose} \xrightarrow{C_x} \text{Cellobiose + Glucose} \xrightarrow{\text{cellobiase}} \text{Glucose}$$

This theory greatly stimulated research and interest in cellulolytic enzymes. Reese and his colleagues continued to screen microorganisms for cellulase production, to optimize media and cultural conditions, and to separate and study the cell free enzymes and their interactions with cellulose. Related studies were being carried out by Geoffrey Halliwell at the Rowett Research Institute, Kendall King at Virginia Polytechnic Institute, Kazutosi Nisizawa at the Tokyo University of Education and Keith Selby at the Shirley Institute. Ellis Cowling at the Yale School of Forestry was studying the structural features of cellulose that influence its susceptibility to enzymatic hydrolysis and Nobuo Toyama at Miyazaki University was investigating the uses of cellulases in extraction of cell contents and other food applications. These workers and others participated in an ACS symposium organized and chaired by Elwyn Reese in Washington, DC, in 1962 (2). The gist of these papers was to reaffirm the multiplicity of cellulolytic enzymes

and their synergistic action on insoluble cellulose, and a growing recognition that various species of Trichoderma were the best sources of active cellulases.

In 1968 Katz and Reese (3) reported the production of 30% glucose from a 50% slurry of heated ball milled cellulose (Figure 1). This was a test tube experiment (one ml) with a high cellulase concentration, and supplemented with Aspergillus luchuensis β-glucosidase, and incubated for 15 days, but there were no apparent obstacles to scale up and speed up, and for the first time a commercial process appeared feasible. In 1969 Elwyn Reese organized and chaired a second ACS symposium at Atlantic City (4) with many of the same participants as at the 1962 symposium and a few new faces, particularly Karl Erik Eriksson from the Swedish Forest Products Laboratory, Tarun K. Ghose from Natick later to become head of Biochemical Engineering at the Indian Institute of Technology in New Delhi, and J. M. Leatherwood from North Carolina State. The emphasis now was on Trichoderma as a source of active cellulase; on cellulase multiplicity, although now we were talking about endo-β-1,4-glucanases (the old C_x enzymes) and exo-β-1,4-glucanases (not the old C_1 enzymes); and a strong new interest in the role of enzymes in using cellulose for animal feed and as a source of hydrolysis sugar. Ghose reported continuous production of 5% glucose from a 10% slurry of heated milled cellulose in a one liter stirred tank reactor (4). In 1970 Ghose and Kostick devised a system whereby the enzyme and undigested cellulose could be retained in the reactor for further hydrolysis and a clean glucose solution could be continuously removed through an ultrafiltration membrane of 10,000 - 30,000 MW cut off (5, 9). In this system 15% glucose syrups were produced from 30% slurries of heated milled cellulose. The system was simplified and improved by taking advantage of the fact that milled cellulose strongly adsorbs cellulase and the adsorbed enzyme was sufficient to digest the cellulose with no replenishment of enzyme even though sugar solution was continuously removed (6, 13).

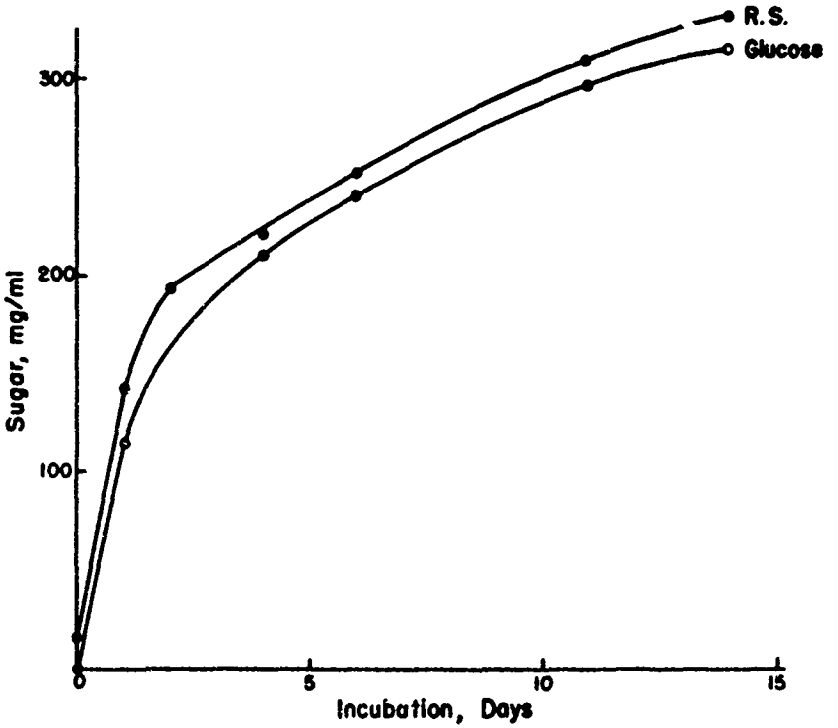


Figure 1. The Production of 30% Glucose Syrup from Heated, Milled Cellulose by Trichoderma reesei Cellulase.

Katz and Reese 1968 (1)

Test tube experiment (one ml) 50% milled cellulose plus cellulase (Trichoderma) and β-glucosidase (Aspergillus).

●——● Reducing Sugar (Dinitrosalicylic Acid)

○——○ Glucose (glucose oxidase)

During the past ten years interest in these applications of cellulase grew rapidly and spread to laboratories all over the world particularly as the rising price and diminishing availability of petroleum directed attention and financial support to the utilization of renewable resources for food and chemicals. Frequent cellulase symposia have been organized in connection with ACS, ASM, and AIChE meetings or as independent forums. Natick had the personnel and the know-how to play a leading role and make major contributions in this development. Mr. Leo Spano became the manager of the program and was able to obtain generous financial support from DA and DOE. He used this to purchase first-class laboratory and pilot plant equipment and to hire a fine group of young microbiologists, chemical engineers, and technicians. In 1975 Natick hosted its own symposium on "Enzymatic Conversion of Cellulose: Technology and Applications" (34) which attracted over 300 attendees, most of them from industry. However, as interest in other academic and industrial laboratories grew and as Natick was increasingly recognized as "the roots and most of the branches of the tree of cellulase knowledge" (Elmer Gaden, personal communication) by DOE and the scientific community, the Army decided to phase out the program and today most of our staff has retired, resigned, or been reassigned to other projects. In this report we will try to highlight the Natick contributions during those exciting years and their impact on the present economics of a commercial process.

Research: Results and Conclusions

1. Microbial Strains

Although we continue to investigate all promising microbial cellulase producers at Natick, we have not yet found the equal of Trichoderma reesei. The parent strain QM6a was isolated from a deteriorated shelter half from Bougaineville Island at the end of World War II (40). This strain was originally identified as a T. viride but in 1977 it was recognized as a new species belonging to the T. longibrachiatum aggregate, but morphologically distinct from it and it was named Trichoderma reesei, Simmons (53), in honor of Elwyn Reese. Many enhanced cellulase mutants have been developed from this strain at Natick (7, 24, 59, 87) and by Bland Montenecourt and Douglas Eveleigh at Rutgers (Figure 2). The differences between the mutants and the wild strain are only quantitative; i.e., the mutants produce higher levels of cellulase than the parent strain, but the composition and properties of the enzyme complex are similar for all regardless of the strain or the inducing substrate and the relative proportions of endo- and exo-β-glucanases show no marked changes (65, 77, 85, 86) (Table 1) (Figure 3). The advantages of T. reesei as a source of cellulase are (a) that it produces a complete extracellular cellulase with all of the components required to hydrolyze crystalline cellulose and (b) that very high yields of cellulase protein are attainable.

2. Growth and Enzyme Production

Growth of Trichoderma reesei and its relation to enzyme production has been investigated on agar plates, in shake flasks, in batch cultures in 10 L to 300 L fermentors, in fed batch cultures (400 ml) and continuous cultures (10 L) on glucose, lactose and cellulose (22, 25, 33, 38, 41, 44, 45, 48, 49, 50, 57, 60,

Figure 2. Trichoderma reesei Mutants

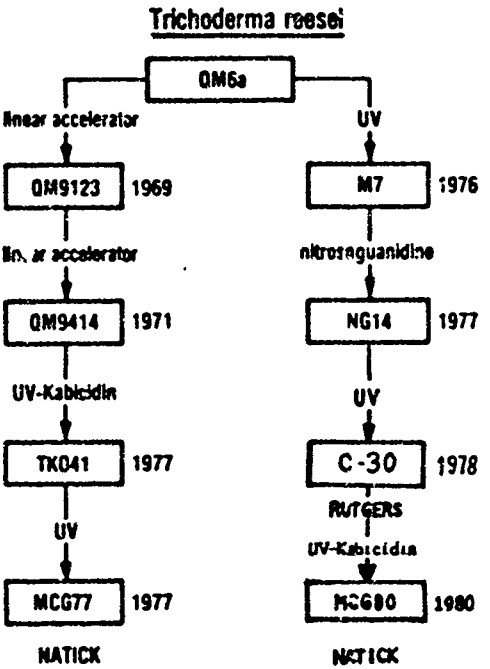


Table 1. Effect of Strain and Substrate on Cellulase Production by Trichoderma reesei

Strain	Substrate 6%	Soluble Protein mg/ml	Filter Paper Cellulase u/ml	CMC Cellulase u/ml	Cellobiase u/ml
QM6a	Lactose	0.9	0.3	8.	NT
	Ball Milled Pulp	9.8	0.6	76.	NT
	FB Cotton	7.4	5.0	88.	NT
QM9414	Lactose	0.9	0.4	10.	0.04
	Ball Milled Pulp	8.7	5.0	178.	0.6
	FB Cotton	13.6	10.0	109.	NT
MCG77	Lactose	3.7	1.8	36.	0.4
	Ball Milled Pulp	9.6	7.4	125.	1.6
	FB Cotton	16.2	10.7	104.	NT
Rutgers NG14	Lactose	NT	NT	NT	NT
	Ball Milled Pulp	14.4	9.0	NT	1.0
	FB Cotton	21.2	14.8	133.	NT
Rutgers C30	Lactose	5.5	1.5	27.	0.07
	Ball Milled Pulp	17.8	11.9	116.	1.0
	FB Cotton	20.6	13.6	181.	NT

Ball Milled Pulp = 200 mesh

Andreotti et al. 1981 (85)

NT = Not tested

FB Cotton = Absorbent Cotton processed for one minute (10 mil gap) on Farrel Birmingham 2 Roll Mill (67).

Cultures grown in fermentors at 28°C with pH controlled not to go below 3.0.

Enzyme units = micromoles glucose produced per minute in standard assay (37).

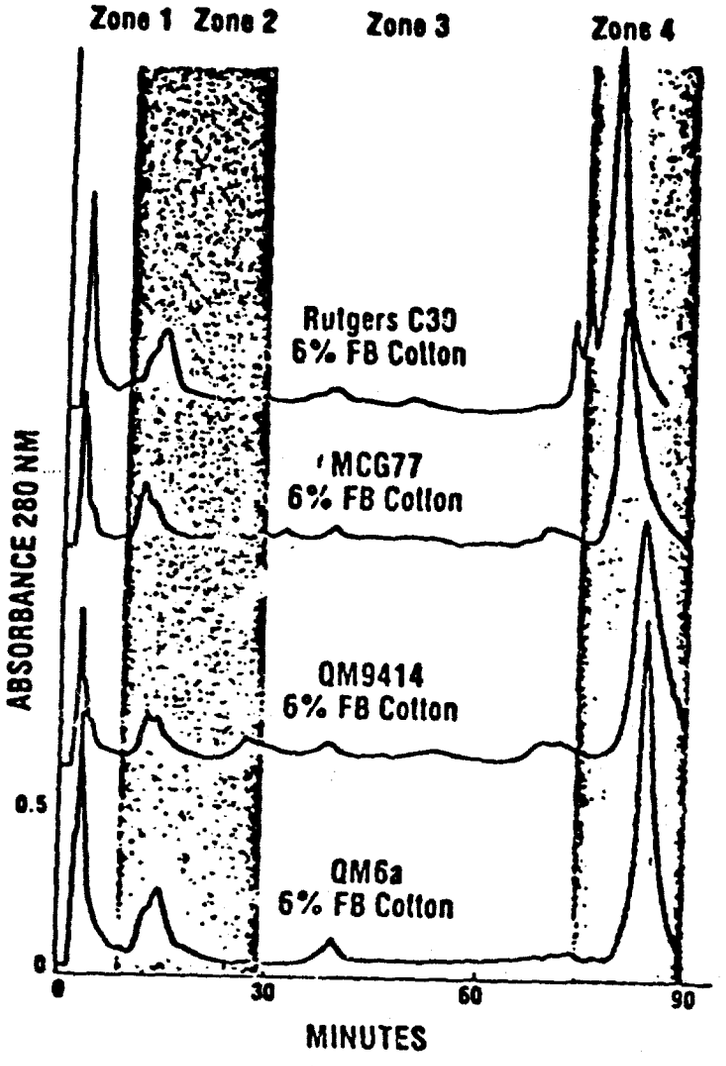


Figure 3. Separation of Endo- and Exo-β-Glucanases of Trichoderma reesei by HPLC. Bissett et al. 1981 (86).

Cultures Grown on 6% Compression Milled (FB) Cotton

Zone 1 β-Glucosidase + Endo-β-Glucanase A

Zone 2 (Shaded) Exo-β-Glucanase A

Zone 3 Endo-β-Glucanase B

Zone 4 (Shaded) Exo-β-Glucanase B

69, 70, 75, 84, 85, 88). T. reesei grows readily in submerged culture on inorganic salts including ammonium as a nitrogen source and any of a wide variety of carbon sources. Other organic growth supplements are not required. Maximum specific growth rates on glucose are 0.12 - 0.24 g/g per hour under the optimum conditions, pH 4.0 and 35°C. Cell yields are 0.4 - 0.5 g/g glucose, 32 - 35 g/mmole O_2 and 10 - 15 g/g nitrogen consumed. All available strains are inducible by cellulose, lactose, or sophorose and all are repressible by glucose and cellobiose. Cellulase can be induced by growth of T. reesei on cellobiose provided initial concentration of the cellobiose is high (1%). However, cellulase appears only after the cellobiose has been consumed and cellobiose is not an inducer for washed mycelium.

The regulatory role of sophorose (2-O-β-D-glucopyranosyl-D-glucose) in cellulase induction by T. reesei has been investigated in washed mycelium experiments (Figure 4). At 10^{-5} M sophorose is a powerful inducer of endo- and exo-β-1,4-glucanase but it does not induce cellobiase (71). Cellobiase in T. reesei is constitutive and intracellular. The constitutive level is about 0.2 - 0.3 international units per mg of mycelium. Higher levels up to one unit per mg are inducible by methyl-β-glucoside (97). Cellobiase also hydrolyzes sophorose (K_m 1.4 x 10^{-3} M). Sophorose may be the natural inducer of cellulase. Cellulases and cellobiase are transferases as well as hydrolases so various β-glucosides, including sophorose, may be produced as by-products during enzymatic hydrolysis of cellulose. When hydrolysis sugars were used as the carbon source in a fed batch experiment, cellulase was produced (84). Synthetic hydrolysis syrups containing equal levels of glucose, cellobiose, and xylose did not induce cellulase (84). When the concentration of sophorose falls to very low levels it would be advantageous for the fungus if cellobiase production were repressed, and in fact this happens at 10^{-7} M sophorose (97).

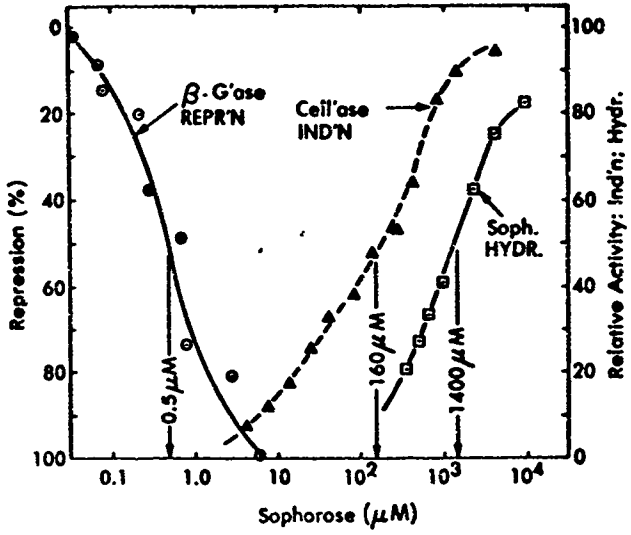


Figure 4. Effect of Sophorose Concentration on its Role in Enzyme Production by Trichoderma reesei QM6a. Sternberg and G. Mandels 1980 (80).

● —— ● Repression of β-Glucosidase Induction (Methyl-β-Glucoside Inducer)

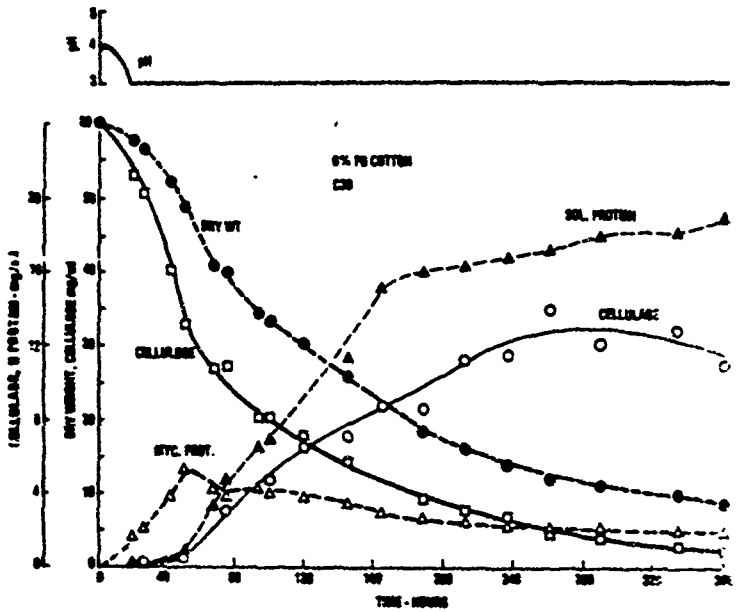
▲ ---- ▲ Induction of Cellulase

□ —— □ Sophorose as a Substrate for β-Glucosidase, K_m = 1.4 mM

Lactose is a good inducer of cellulase, particularly for some mutant strains (85, 87) and has proved useful in continuous culture studies since it is easier to feed and monitor consumption of a soluble substrate (69). The highest yields of cellulase are usually produced on cellulose (Figure 5) and this will probably be the substrate of choice for industrial production of the enzyme. The optimum conditions for cellulase production pH 2.8, temperature 28°C, are somewhat lower than the growth optima. However cellobiase is unstable at low pH. If pH is held at 5.0 and temperature reduced to 22°-25°C cellobiase yields can be appreciably increased without greatly reducing the cellulase titre.

Cellulases are extracellular and only very low levels are ever found associated with the mycelium. Although most of the enzyme appears after growth is complete and most of the substrate has been consumed, cellulase secretion ceases when the inducing substrate (cellulose, lactose, sophorose) is depleted. The highest yields of cellulase have been achieved on two roll milled cotton or Avicel. These substrates are sufficiently susceptible to hydrolysis to permit initial rapid growth and also contain resistant fractions that are very slowly consumed, thereby prolonging enzyme secretion. The highest reported yields of cellulases are about 15 international filter paper units per ml (Table 1). Even higher yields have been attained in unpublished experiments using a new hyperproducing strain of T. reesei, MCG80 (B. Gallo, personal communication). Enzyme level is proportional to initial cellulose concentration (45, 48). Because of the low specific activity of cellulase these yields represent very large quantities of cellulase protein - over 20 g per liter from 6% two roll milled cotton (Table 1). This is a remarkable yield of cell free microbial protein. On more susceptible substrates yields are somewhat reduced, but productivities of over 200 mg of cellulase protein per liter per hour have been achieved. The amino acid composition is that

Figure 5. Fermentation Profile of Trichoderma reesei Rutgers Strain C30 Growing on 6% Compression Milled Cotton



FB = Farrel Birmingham 2 Roll Mill

Cellulase = International Filter Paper Cellulase Units/ml

Andreotti 1979 (unpublished data)

of a "complete" protein without deficiencies in any essential amino acids (57) and this protein may have value as a meat extender competitive with soy protein.

3. Enzyme Properties, Analysis and Assay

The multiplicity of cellulases was first postulated and demonstrated at Natick (1), but the careful biochemical separation, purification, and characterization of T. reesei cellulase components has been mostly the work of biochemists at other laboratories, particularly Ross Brown at Virginia Polytechnic Institute, Goran Pettersson at the University of Uppsala, and Tom Wood at the Rowett Research Institute in Great Britain. A useful Natick contribution was the development of a rapid HPLC procedure suitable for rapid separation and quantification of the cellulase proteins in a crude enzyme preparation (65) (Figure 3). About 30% of the cellulase protein is associated with the endo-β-1,4-glucanases (EC3-2-1-4) that randomly hydrolyze the internal linkages in the cellulose chain, and about 70% of the cellulase protein is associated with the exo-β-1,4-glucanases (cellobiohydrolases) (EC3-2-1-91) that remove cellobiose groups sequentially from the non reducing chain ends. Synergistic action by the endo- and exo-β-glucanases is required for hydrolysis of insoluble cellulose. These enzymes are product inhibited by cellobiose. Only about 1% of the protein in a Trichoderma cellulase preparation is cellobiase (EC3-2-1-21) but this is adequate to hydrolyze the cellobiose produced by cellulase action under growth conditions where glucose does not accumulate. However, cellobiase is strongly product inhibited by glucose (43, 61, 97) so cellobiase levels are not high enough to obtain maximum rates of cellulose hydrolysis in a hydrolysis reactor.

The measurement of cellulase activity is complicated by the multiplicity and synergistic action of the enzymes and also by the variability of the substrate. The cellobiase enzymes are measured by the release of glucose from soluble substrates

cellobiose, short chain cellodextrins, alkyl (β-Methyl) or aryl (Salicin, p nitrophenyl-β-D-) glucosides. For cellulase work cellobiose is the preferred substrate. Purified T. reesei cellobiase has about 150 international units per mg of protein. Endo-β-glucanases are measured on soluble cellulose derivatives of low degree of substitution such as carboxymethyl cellulose by either reduction in viscosity (due to reduced chain length) or the release of reducing sugars. T. reesei crude cellulase has about 20 international CMC'ase units per mg of protein. For insoluble cellulose the specific activity is lower, usually less than one international unit per mg of protein in standard assays. Actual value depends on the nature of the substrate (degree of crystallinity, pretreatment), cellulose concentration, available surface, and the extent of hydrolysis. The filter paper assay introduced at Natick (36) (Figure 6) has been widely adopted as a practical measure of total cellulase action on insoluble cellulose. Because of the low specific activity of T. reesei cellulase preparations, the total protein is usually a good measure of the quantity of enzyme. For process development enzyme must be evaluated under realistic conditions, 10-20% substrate concentration and 24-48 hours hydrolysis (92) (Figure 7).

The optimum stability and activity of T. reesei cellulase under process conditions are pH 4.8, 50°C. The cellobiase (Salicin) and endo-β-glucanase (CMC) are more stable than the exo-β-glucanase (filter paper, cotton) (Figure 8) but even after 28 days incubation of enzyme in the absence of substrate at 50°C, pH 4.0-6.0, significant levels of saccharifying cellulase are retained (Figure 9). In general the exo-β-glucanases are more sensitive to heat, pH extremes, and chemical inhibitors than are the endo-β-glucanases and β-glucosidases (61, 76). These enzymes are also sensitive to shaking (shear) inactivation which occurs at the air liquid interface (74). This can be alleviated by increasing enzyme concentration or by addition of fluorocarbon surfactants or high molecular weight

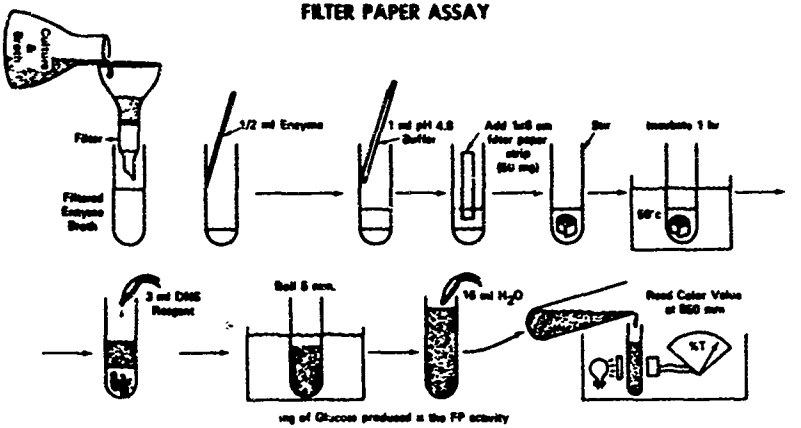


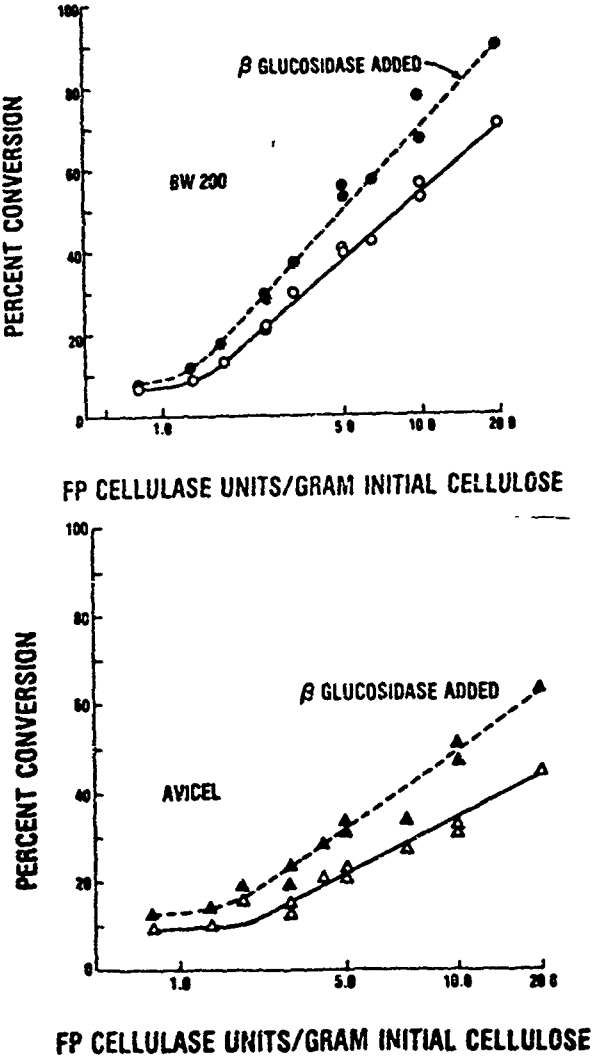
Figure 6. The International Filter Paper Cellulase Unit is the amount of enzyme that produces one micromole (0.18 mg) of reducing sugar as glucose per minute. The Natick FP unit is determined by running a dilution curve to find the quantity of enzyme solution (filtrate) that gives 2 mg of "glucose" in the above test in one hour (0.185 units).

FP units/ml = 0.185/ml enzyme to give 2 mg "glucose".

Mandels et al. 1976 (36)

Figure 7. Effect of Enzyme Substrate Ratio and of Added β-glucosidase on Percent Conversion. Mandels et al. 1981 (92)

o —— o BW200 5-15% Ball Milled Pulp
● ··· ● BW200 - β-glucosidase added to equal FP cellulase U/ml
Δ —— Δ Avicel 5-15% Microcrystalline Cellulose
▲ ··· ▲ Avicel - β-glucosidase added to equal FP cellulase U/ml
Hydrolysis at pH 4.8, 50°, shaken, for 48 hours



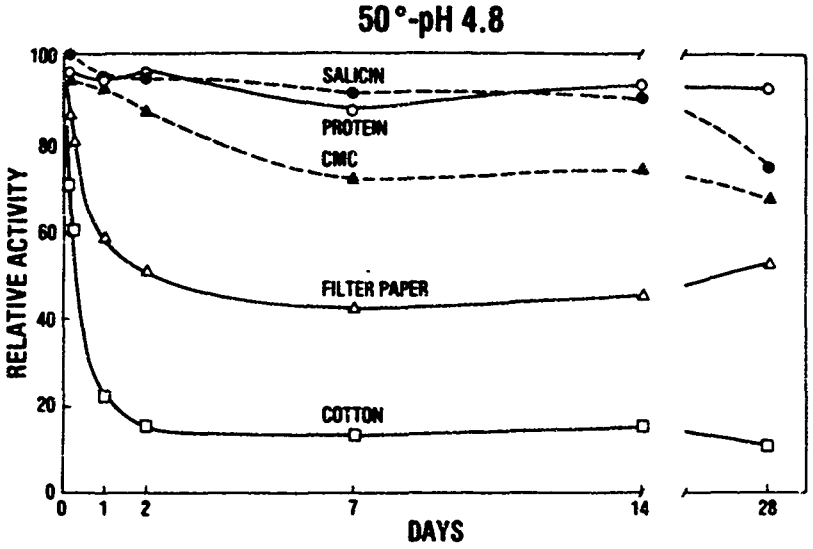


Figure 8. Stability of Trichoderma reesei Cellulase

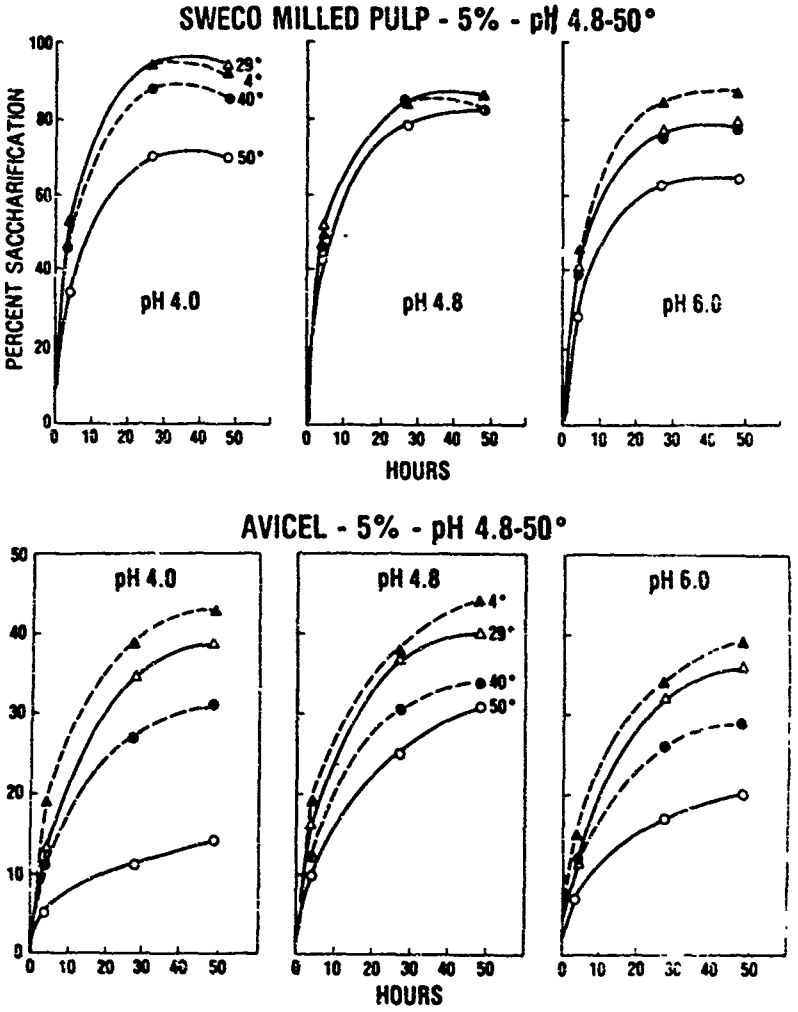
Trichoderma reesei QM9414 cellulase broth from a 6% cellulose culture diluted 1:4 and incubated at 50°, pH 4.0, 4.8, and 6.0 in the absence of substrate. Residual activity measured at pH 4.8, 50°. Values for shaken and unshaken flasks averaged.

Reese and Mandels 1980 (76)

Figure 9. Saccharifying Power of Trichoderma reesei after 28 days incubation in the absence of substrate under indicated conditions.

Mandels et al. 1978 (67)

Saccharification at pH 4.8, 50°C.



polyethylene glycols in low concentration. These have been proposed to act by reducing the surface excess of protein (72, 93).

4. Hydrolysis Substrates

Many of the fundamental studies on cellulase have been carried out on pure cellulose substrates. These are too expensive for use in a practical process. More than 200 samples of cheap (or no cost) cellulosic wastes including paper mill wastes, sludges and pulps, assorted wood wastes, fiber fractions from municipal trashes treated by Black Clawson, ADL, and Bureau of Mine processes, various grasses, hays, straws, bagasse, corn stover, grape pulps, skins and seeds, other agricultural wastes, newspaper and other waste paper, etc., have been evaluated as process substrates at Natick (19, 20, 31, 32, 81). An ideal substrate would be cheap, available in large quantity the year round, and would require little or no pretreatment. Most of the substrates evaluated here required some pretreatment, but several then gave about 50% yields of soluble sugars (predominately glucose, cellobiose, xylose) after 24-48 hours hydrolysis. The most promising substrates were paper mill wastes and municipal wastes. In the long run wood chips or other "energy crops" would be required for a large scale process.

5. Pretreatment

For most substrates some pretreatment to reduce crystallinity and increase accessibility of the cellulose is required if good rates and extent of hydrolysis are to be achieved. Various chemical and physical procedures to accomplish this have been widely investigated. Very reactive cellulose has been produced by George Tsao and colleagues at Purdue University by dissolving in Cadoxen, CMCS (NaOH, Fe tartrate, citrate), H_2SO_4 or other solvents and then reprecipitating

and washing. At Natick we believe that economic and pollution control constraints will mandate recovery of the solvent (a formidible operation since 4-5 grams of solvent are required to dissolve one gram of cellulose) and therefore this approach is not economic. Dilute acid pretreatment (Grethlein, Dartmouth) and steam explosion (Noble, Iotech) are effective pretreatments for some, but not all substrates. The Natick approach has concentrated on ball milling (3, 5, 9, 12, 19), attritor milling, and particularly two roll (compression) milling (55, 79, 81, 96, 98) as the most widely applicable, effective, and economic pretreatment to reduce crystallinity and increase enzyme reactivity (Table 2).

Compression milling has been shown to be a rapid pretreatment method which is effective on a diverse array of cellulosic biomass, yields a product of high bulk density, and consumes relatively low amounts of energy (55). The enzymatic hydrolysis yield for any given substrate is directly related to the specific energy input which can be controlled by milling time. For newspaper or the cellulose fraction of municipal waste the specific energy input for 55% sugar yield is estimated as 0.3 KWh/lb substrate and the additional yield (over the untreated substrate) is 453 g sugar/KWh energy input (81). Pretreatment efficiency was significantly improved by adjustment of feedstock moisture content prior to milling and/or increasing the roll pressure on the feedstock to reduce specific energy input for 48% sugar yield from newspaper to 0.2 KWh/lb substrate (98). Enzymatic hydrolysis data from a commercial scale run (6 tons/hr/pass) of moist (24%) newspaper correlated well with laboratory scale data, but with a 26% reduction in energy consumption (98). The most important effect of compression milling on cellulose is a decrease in crystallinity. Degree of polymerization is also decreased and accessibility to water vapor and iodine is increased (96).

TABLE 2 - EFFECT OF COMPRESSION MILLING PRETREATMENT ON THE ENZYMATIC HYDROLYSIS[1] OF VARIOUS CELLULOSIC SUBSTRATES

SUBSTRATE	MILLING TIME (MIN)	TOTAL REDUCING SUGARS (MG/ML) 4 HR.	24 HR.
"PURE" CELLULOSES			
SOLKA FLOC (sw40)	0	10.5	16.8
	3	28.5	48.1
ABSORBENT COTTON	0	2.5	3.9
	4	15.7	35.3
WASTES			
URBAN WASTE (B. MINES)	0	10.7	18.5
(W/O PLASTICS)	1.5	20.0	30.5
XYLITOL PROCESS	0	3.5	6.7
	4	28.0	35.5
SOFTWOODS			
EASTERN SPRUCE	0	2.0	3.8
	4	14.8	22.4
WESTERN HEMLOCK	0	1.2	2.0
	4	15.0	19.5
HARDWOODS			
MAPLE	0	1.2	1.6
	5	21.8	28.6
BIRCH	0	1.5	2.4
	4	21.4	25.2
AGRICULTURAL RESIDUES			
CORN STOVER	0	4.6	8.0
	4	20.0	26.5
SUGAR CANE BAGASSE	0	1.6	2.5
	4	13.2	19.6

[1] T. REESEI CELLULASE (GM9414), 19 IU/GM SUBSTRATE, 5% SUBSTRATE SLURRIES, PH 4.8, 50°C Spano et al. 1979 (79).

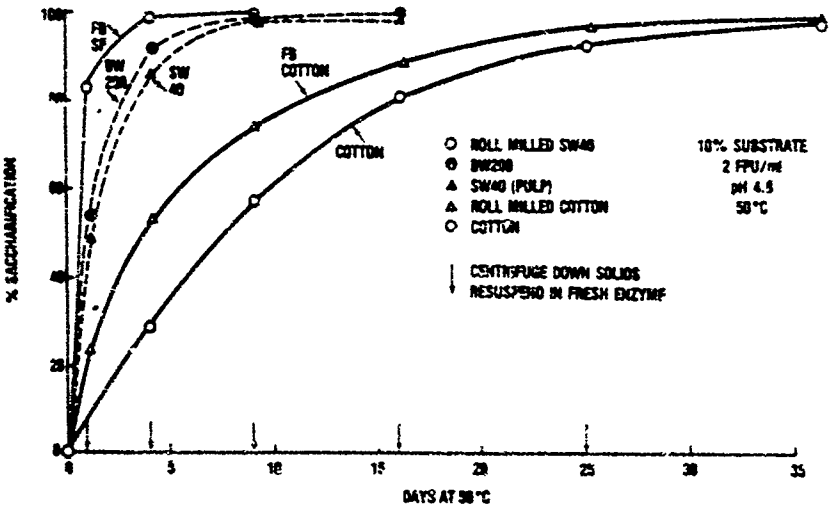
FIGURE 10

ENZYMATIC HYDROLYSIS OF PURE CELLULOSIC SUBSTRATES OF VARYING RESISTANCE SHOWING SUBSTRATE MULTIPLICITY

FB = FARREL BIRMINGHAM TWO-ROLL MILL

SW40 = SF, SOLKA FLOC PULP

BW200 = BALL MILLED PULP



Mandels et al. 1976 (43).

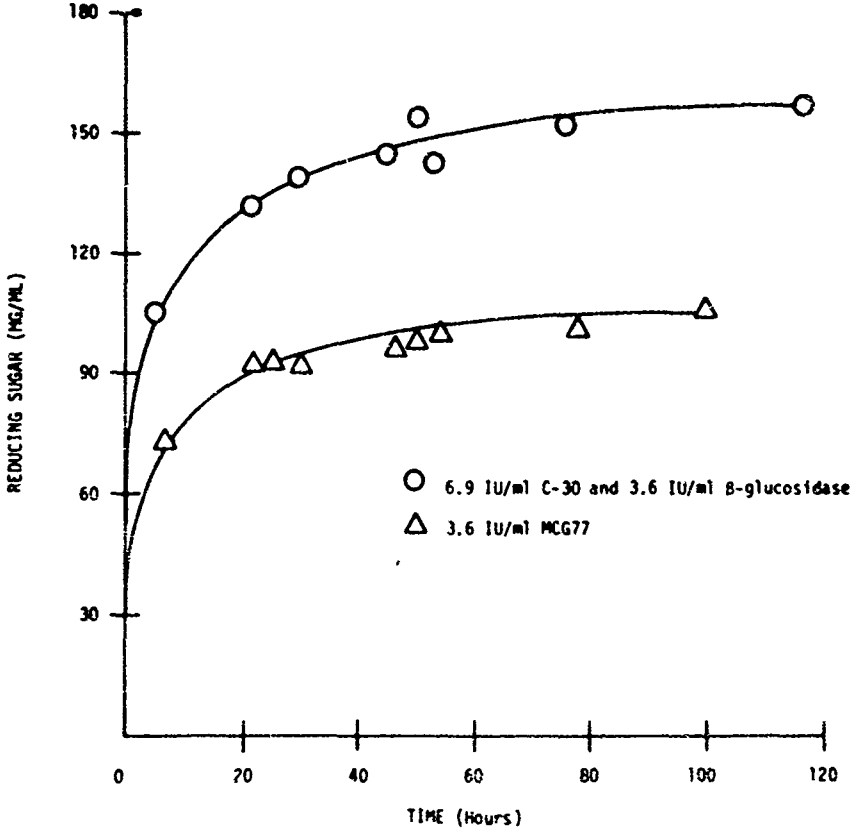
6. Hydrolysis

The value of Trichoderma cellulase is that (a) it is capable of total hydrolysis of accessible cellulose even if that cellulose is crystalline (Figure 10) and (b) it is capable of carrying out extensive hydrolysis of a concentrated cellulose slurry in 24-48 hours yielding sugar solutions of 10% or greater concentration (Figure 1, Figure 5, Figure 11) (3, 5, 8, 9, 10, 12, 13, 19, 23, 33, 39, 46, 56, 61, 83, 92). More than 200 pure and waste cellulose substrates have been tested at Natick and many appear suitable for a practical process (19, 20, 31, 32). Optimum conditions for hydrolysis are pH 4.8, 50°C. Since the amount of sugar produced (or percent conversion) is a function of the enzyme substrate ratio, very large quantities of enzyme are required for extensive conversion. To achieve a 40-50% conversion of a 10-30% slurry in 24-48 hours requires about 10 filter paper units/gram for a susceptible substrate, and about 20 units/gram for a more resistant substrate (Figure 7) (92). Since the specific activity of cellulase on filter paper is only about 0.6-0.7 units/mg protein the requirement is for 15-30 g of enzyme protein per kg of substrate. The enzyme protein requirement for cellulose hydrolysis is about 100 times that for starch hydrolysis.

This high cellulase requirement and the cost of producing the cellulase is the major economic barrier that must be lowered before enzymatic saccharification of cellulose becomes economically feasible. To date the search for new strains and mutants of existing strains of microorganisms has not turned up a cellulase with a significantly higher specific activity than Trichoderma cellulase. The low specific activities and efficiencies of cellulases would appear not to be a "fault" of the microorganism but are inherent in the insoluble and recalcitrant (to hydrolysis) nature of cellulose and the necessity for synergistic action by

Figure 11

EFFECT OF HIGH CELLULASE AND HIGH β-GLUCOSIDASE LEVELS ON THE HYDROLYSIS OF 30% NEP-200 AT pH 4.8 AND 50°C



Blodgett 1931 (Unpublished Data)

several enzymes. As percent conversion increases, hydrolysis rate slows due to increasing resistance of the residual substrate (8, 35), product inhibition (61) and enzyme inactivation (61, 76). These are important problem areas that must be addressed before a commercial process is possible.

Pretreatments (as discussed in section 5) do reduce crystallinity and increase enzyme accessibility, but the above enzyme requirements are for pretreated substrates. Research on economic and effective pretreatments should have a high priority.

The products of cellulase action on cellulose are cellobiose and glucose. Trichoderma cellulase preparations also contain many other enzymes (29, 52) so when crude cellulosic substrates are used, other sugars, particularly xylose are also found in the digests. These sugars are readily identified by high performance liquid chromatography. This procedure, which is now very widely used, was pioneered by J. Palmer at Natick (26, 27). Cellulases, acting on insoluble cellulose, are strongly inhibited by cellobiose. The β-glucosidase (cellobiase) enzymes hydrolyze cellobiose and relieve this inhibition, but the β-glucosidases are in turn inhibited by glucose, so that as glucose accumulates in a saccharification reactor, the activity of the cellobiase is reduced, cellobiose accumulates, and the cellulase activity is inhibited (43). We have not yet learned how to achieve significantly higher levels of β-glucosidase from Trichoderma in a highly productive cellulase fermentation (80, 97). However, alternate sources of β-glucosidase, particularly Aspergillus phoenicis QM329 have been identified (54) and the production of this enzyme in batch (72) and fed batch (82) fermentations has been optimized to give a maximum titre of 115 units/ml and a maximum productivity of 950 units/liter/hour. Addition of supplemental Aspergillus β-glucosidase alleviates product inhibition of cellulase and can reduce the cellulase requirement

for any desired conversion by 50% (Figure 7). Because of the high specific activity of this enzyme it is much less expensive, per unit, to produce than is cellulase. Since the substrate cellobiose is soluble, β-glucosidase can be immobilized. This increases its stability to pH and temperature extremes and permits reuse (58) (Table 3). An alternate way to reduce product inhibition is by use of membrane (5, 9) or adsorption (5, 13) reactors where products are continuously removed or by simultaneous saccharification and yeast fermentation (SSF) to ethanol (62).

Another means of using cellulase more effectively would be to recover and reuse the enzyme from the hydrolysis reactor. Enzymes remaining in the syrups after hydrolysis can be precipitated by tannin or solvents, or recovered by adsorption on fresh substrate or by ultrafiltration. Since cellulases (but not β-glucosidases) are strongly adsorbed by cellulose (6, 51, 92) recovery will be modest unless most of the cellulose has been digested, or means are found to elute enzyme from residual solids. Cellulase can be eluted from Avicel with water but the eluents are dilute, or it can be more successfully eluted with 2-6 M urea, guanidine, or dimethylsulfoxide (95). Further work will be required to develop this important finding for more practical substrates and to test the possibility (and economics) of commercial application. Stabilization of enzymes becomes more critical when recovery is contemplated. The use of additives such as surfactants to increase enzyme stability increases the rate and extent of hydrolysis and the amount of residual enzyme in solution.

7. Process Development and Economics

The technical feasibility of an enzymatic process for the conversion of waste cellulose to glucose has been demonstrated. Natick workers have developed cost analyses (30, 33, 38, 39, 42, 56, 63, 64, 79, 83, 89) (Table 4) for a plant

Table 3

Thermal stability of free and immobilized β-glucosidase

°C	half life (hr) Free	Immob.
55	216	
60	8.6	
65	0.5	252
70	0.04	43
75	-	1.6
80	-	0.07

Enzymes were incubated at the designated temperatures in 0.05M citrate buffer pH 4.8 and then assayed for retention of activity.

Bissett and Sternberg 1978 (58).

Table 4

COST ANALYSIS - ETHANOL FROM CELLULOSE

Based on 25 x 10^6 gallons/year from 5 x 10^8 Kg Urban Waste*

(Cyclone → Light Fraction = 3.7 x 10^8 Kg Hydrolyzable Substrate)

24 Hour Hydrolysis → 1.67 x 10^8 Kg Glucose

Cost Factor	Assumption	$/Gallon 95% Ethanol
Substrate*	30% Charge to Reactor	0.13
Pretreatment	Two Roll Mill	0.22
Enzyme	10 FPU/g Substrate	0.45
Hydrolysis	45% Yield (10% syrup)	0.13
Ethanol Production	40% Yield from Sugar	0.28
Total Factory Cost	No Credit for By-products	1.21

Enzyme Productivity = 125 Filter Paper Cellulase Units/Liter/Hour
Enzyme Utilization Efficiency (24 Hours) = 13%
*Separated Fiber Fraction at $6/Ton

Spano et al. 1979 (79)

based on the Natick technology that would produce 25 x 10^6 gallons of 95% ethanol per year (65,493 gallons per day) from separated urban waste using the cellulose rich fiber fraction. The plant would require 4.95 x 10^5 tons per year or 1356 tons per day of this substrate derived from about 3400 tons per day of urban waste. The substrate would be pretreated by 2 roll milling and hydrolyzed as a 30% slurry with 10 filter paper cellulase units per gram at pH 4.8, 50°C to yield 1.67 x 10^5 tons of glucose per year. The glucose would be fermented to ethanol by yeasts. Overall yields are 340 kg of glucose yielding 50 gallons of ethanol per ton of the fiber fraction or 20 gallons of ethanol per ton of municipal waste. At 10 filter paper cellulase units/g this plant would require 4.95 x 10^{12} cellulase units or (at 0.6 FPU/mg protein) 8.25 x 10^6 kg of enzyme protein per year or 300 g of enzyme protein per gallon of ethanol. Factory cost of the ethanol was estimated at $1.21 per gallon in 1979 (79) when ethanol sold for $1.10 per gallon. Process improvements and credits for by-products have resulted in significant reductions in this cost estimate. However it would appear that the process is not yet economically viable without a very cheap substrate and some subsidy. The successful gasohol from corn program is heavily subsidized by state and federal governments.

8. Outlook, Future Developments

Although the Natick program on cellulose conversion has terminated, extensive development based on the Natick process continues at Gulf Oil, University of Arkansas, University of California, Purdue University and Rutgers University, and in many foreign countries including Brazil, Canada, China, Finland, France, India, Italy, Japan, Korea, Mexico, New Zealand, Russia, South Africa and Taiwan. The Natick group continues to receive many requests for information, permission to visit us, and invitations to lecture or present courses. Interest in cellulose

conversion remains very strong because the product, glucose, is a stable and useful compound readily separated from the digest, process development is well advanced, and because cellulose is the only renewable resource available in sufficient quantity to support large scale industrial processes. Increasing utilization of starch much beyond the present levels would create too many problems of rising food prices, reduced grain exports, and soil erosion as marginal land is brought under cultivation. Furthermore, because petroleum is consumed in producing pesticides, fertilizers, and farm equipment, and in growing and processing grain, the net reduction in petroleum useage when grain is converted to ethanol is disappointing. Lignin is the other abundant renewable carbon compound but so far no one has developed a method for producing useful stable intermediates from lignin degradation. Most of the cellulose processes being developed assume that residual lignin will be burned to provide some of the process energy. More profitable uses for lignin will further insure the economic success of the cellulose conversion process.

Critical research areas remain to be investigated before the process is ready to stand on its own feet: particularly (a) increasing production and reducing cost of the enzyme, (b) better pretreatments that are both efficient and economical, and (c) reducing the enzyme requirement either by increasing the specific activity, by enzyme stabilization, or by enzyme recovery and reuse. There also remain challenging fundamental research problem areas in elucidating the synergistic interactions of a multiple enzyme system with its complex insoluble substrate and the biological controls for synthesis and secretion of extracellular enzymes such as Trichoderma cellulase. We hope that Natick scientists can continue to play a role in the further development of microbial cellulases and their applications.

Cellulose Bioconversion
Natick Contributions and Milestones

1. C_1 C_x concept. Cellulase is not a single enzyme, but a mixture (1950).

2. As a result of screening thousands of organisms, Trichoderma reesei was identified as an excellent source of cellulolytic enzymes (1955).

3. Total hydrolysis of crystalline cellulose by cellulase was achieved (1964).

4. Separation of cellulase components required for hydrolysis of crystalline cellulose, and demonstration of synergism after recombination (1964).

5. Study of cellulase inhibitors including natural inhibitors from plant sources. Persimmon inhibitor active at 1 p.p.m. (1965).

6. Thirty percent glucose syrups were produced from milled cellulose pulp demonstrating the possibility of a commercial process (1968).

7. Continuous production of 5% glucose syrups from a stirred tank reactor (1969).

8. Membrane reactor for continuous production of glucose syrups with retention and reuse of enzyme (1970).

9. Use of surfactants to increase yields of cellulase and many other enzymes (1970).

10. Mutation of Trichoderma reesei to strains that produce higher levels of cellulase (1970) (1977-1981).

11. Adsorption reactors and cellulose columns for continuous production of glucose syrups with retention and reuse of enzyme (1971).

12. Investigation of over 200 wastes as hydrolysis substrates, and saccharification of many of these in stirred tank reactors (1975).

13. Development of an HPLC procedure for analysis of hydrolysis sugrar (1975).

14. Pilot plant cellulase production and cellulose saccharification at 300 liter scale (1975).

15. Development of two roll compression milling as a promising pretreatment for hydrolysis substrates (1977).

16. Role of β-glucosidase in removing inhibitory cellobiose. Enhancement of saccharification by addition of supplemental β-glucosidase (1977).

17. Coupled saccharification and ethanol fermentation by Candida utilis as a means of reducing product inhibition (1977).

18. Optimization of fermentation and use of new cellulase mutants to produce over 2% extracellular cellulase protein from 6% cellulose (1977).

19. Studies on adsorption and desorption of cellulases and their roles in hydrolysis and enzyme recovery (1977 - 1981).

20. Immobilization of Aspergillus luchuensis β-glucosidase and its use in saccharification (1978).

21. Development of a rapid HPLC procedure for analysis and quantification of cellulase proteins (1978).

22. New investigations on constraints and inhibitory factors in saccharification, and use of surfactants to protect cellobiohydrolase from shear inactivation (1978 - 1981).

23. Studies on induction of cellulases and β-glucosidase and the role of sophorose in controlling enzyme production in Trichoderma reesei (1979 - 1981).

24. Development of fed batch and continuous cultures of Trichoderma reesei on soluble substrates (lactose, enzyme hydrolysis sugars) to increase enzyme productivity and to estimate quantitative data (yield and maintenance constants, specific nutrient requirements, specific enzyme productivity) necessary for economic evaluation (1979 - 1981).

25. Optimization of batch hydrolysis conditions to yield 10% glucose syrups from milled waste in 8 hours (1979 - 1981).

26. Complete economic analysis of ethanol from celllulose process (1979 - 1981).

Acknowledgments

Personnel actively associated with the research on cellulose conversion are listed on pages 37 and 38 and also appear as authors on the attached list of Natick Publications. Dr. Gabriel Mandels and Dr. Frederick Robbins, although not in the Enzyme and Biochemical Engineering Group, sometimes participated in the cellulase research and frequently contributed scientific advice and/or assistance in preparation of manuscripts. Dr. Fred Oesterling, former Deputy Technical Director at Natick, was our first and strongest administrative supporter. Dr. Dale Sieling, former Technical Director at Natick; Dr. Hamed El Bisi, Deupty Technical Director; and Dr. S. David Bailey, Director of the Science and Advanced Technology Laboratory also gave good administrative support. Dr. Elmer Gaden of the University of Virginia, Dr. Arnold Demain of the Massachusetts Institute of Technology, and Dr. Karl Smiley of the U.S.D.A. Northern Regional Research Laboratory at Peoria served as successive chairmen of our National Academy of Sciences/National Research Council Advisory Board and provided valuable technical guidance and suggestions.

Personnel Associated with the Cellulose Conversion Work

1. Regular Staff

Alfred Allen	Chemical Engineer	through 1981
Raymond Andreotti	Engineering Technician	"
Curtis Blodgett	Chemical Engineer	"
Richard Erickson	Engineering Technician	"
Benedict Gallo	Microbiologist	"
Charles Macy	Chemical Engineer	"
Mary Mandels	Microbiologist	"
John Medeiros	Chemical Engineer	"
Robert Mortensen	Engineering Technician	"
Elwyn Reese	Microbiologist	"
Charles Roche	Biological Sciences Technician	"
Leo Spano	Chemical Engineer	"
Frank Snyder	Chemical Engineer	"
Thomas Tassinari	Chemical Engineer	"

Robert Andren	Chemical Engineer	through 1976
Edward Black	Chemist	through 1977
Paul DiLuca	Biologist	" 1977
John Nystrom	Chemical Engineer	" 1977
Sheila Dorval	Biologist	" 1978
Philip Hall	Physical Sciences Technician	" 1978
Frank Bissett	Chemist	" 1980
David Sternberg	Microbiologist	" 1980

2. NAS/NRC Fellows and Other Visitors

Andrew Huang	Chemical Engineer	1972 - 1974
Chul Kim	Chemical Engineer	1972 - 1974
James Palmer	Biochemist	1974 - 1975
Steven Meyers	Microbiologist	1975 - 1977
Nicolai Peitersen	Bioengineer	1975 - 1977
Dewey Ryu	Bioengineer	1978 - 1979
C. Patrick Dunne	Biochemist	1980 - 1981
James Shieh	Microbiologist	1980 - 1981

3. Summer Helpers (Students)

Felix Losco	1974
Kathy Fallow	1977
Judy Dorr	1978
Pam Robinson	1978
Paul Shea	1978
Cathy Carbone	1980
Pat Farrel	1980
Robert Lelievre	1980
Tracy Walker	1980, 1981
Caryn Mee	1981

4. Military

Dixon Brandt	1971 - 1973
Lloyd Hontz	1971 - 1973
Dave Boger	1973 - 1974
Keith Kornuta	1974 - 1975
Ron Acuff	1975 - 1977
Joy Loehr	1977
Vijayakumar Pamidimukala	1977 - 1978
Joyce Dempsey	1978 - 1979
Marty Foncello	1978
Rovelma Hudson	1978 - 1980
Bob Trainer	1978
Bill Clark	1981

5. Secretaries

Edith Blodgett	through 1981
Beverly Grant	" 1981
Charlene Clebda	" 1976
Ruth Saleson	" 1976
Arlene Parelli	" 1977

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